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A protocol for flow cytometric determination of expected chromosome number of *Brassica juncea* L. introgression lines

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Abstract

Flow cytometry was used to estimate the amount of DNA in nuclei of a set of *Brassica juncea* introgression lines carrying genomic segments from *B. fruticulosa*. *Solanum lycopersicum L.* cv. 'Stupicke´ with 2C nuclear DNA content of 1.96 pg was used as an internal reference standard. The 2C DNA content of 2.440 pg was worked out for the standard, *B. juncea* cv. RLC- 1. The 2C DNA content of *B. juncea* introgression lines ranged between of 2.248 pg (AD4K2-196) and 2.538 (AD3L-526, AD3L-459). Increase in the genome size as compared to the standard *B. juncea* parent, RLC 1 may indicate the addition of alien chromatin from *B. juncea*. A reduction in genome size may indicate the chromatin loss. Results indicated a high correlation (0.84) between the relative nuclear DNA content and the expected chromosome number. The studies showed that flow cytometry can be effectively used to gain more insight into the variation and transmission of DNA content within the alien introgressed populations of crop *Brassica* species.

Key words: *Aneuploidy, C value, DNA content, rapeseed-mustard*

Introduction

Alien introgression is now an important plant breeding technique to deploy wild alleles for combating biotic and abiotic stresses. To expedite the process of alien introgression and to reduce linkage drag, there is always a need to study a large number of introgressed progenies for chromosome number and meiotic stability. This requires laborious and time consuming cytological analysis. Flow cytometry is an attractive alternative. It is a potent procedure of quantitative single-cell analysis, and it prevents the cost and time involved in conducting cytological examinations of a large number of samples. Flow cytometry first became important during late 1970s for analyzing DNA content in human cells. Technique was later extended to include plant cells during the early 1980s. Flow Cytometry is now the method of choice for ploidy analysis because analysis is rapid, representative and large number of nuclei can be measured in a short time (Dolezel *et al.*, 2007). Like all eukaryotes, cell growth and division in angiosperms is a cyclical process. Howard and Pelc (1953) divided the time between one mitosis and the next into three phases *viz*., G1, S sand G2. During the period of cell growth (G1 phase) a diploid cell has 2C (C=DNA content of the haploid set of chromosomes) nuclear DNA content. Duplication of the nuclear genome occurs during the S phase when the DNA contents doubles to 4C. S phase is followed by a second phase of cell growth termed G2 phase during which the DNA content is maintained at the 4C level. This is followed by mitosis (M phase) during which the cell divide to produce two daughter cells with 2C DNA content. Coefficient of variation usually ranges between 1-10%. Nuclear DNA content of over 100 plants species has been measured by flow cytometry of isolated nuclei stained with Propidium iodide (http://data.kew.org/cvalues/introduction.html). Van Tuyl *et al*. (1989) used the method to analyse the ploidy levels of pollen nuclei. *Arabidopsis* shows developmentally regulated multiploidy with a 2C nuclear DNA content of 0.30 pg. Tomato has a 2C value of about 2.0 pg whereas 2C values for *B. juncea*, *B. napus* and *B. nigra* were 2.29, 2.34 and 0.97, respectively (Arumuganathan and Earle, 1991). Johnston *et al.* (2005) studied the Genomic

characteristics of the species of the Brassicaceae and reported 1C nuclear DNA content for *B. carinata*, *B. juncea*, *B. napus*, *B. nigra*, *B. oleracea* and *B. rapa* as 1.308, 1.092, 1.154, 0.647, 0.710 and 0.539, respectively.

In this manuscript, we report the standardization of the flow cytometry method to infer DNA content in a set of *B. juncea* introgression lines carrying genomic fragments from *B. fruticulosa*. All these introgression lines had normal euploid chromosome number expected for *B. juncea*. DNA content of each addition line was determined by comparison with internal or external reference. It was expressed as a difference with respect to the original parental line.

Materials and Methods

Plant material: The experimental material comprised a set of 36 *B. juncea* introgression lines having more than 90 percent pollen grain stainability and lower aphid infestation index, which are indicative of introgression of aphid resistance/ tolerance from wild parent, *B. fruticulosa.*

Isolation and staining of nuclei: A reagent kit, Partec CyStain UV precise P, was used for nuclei extraction and DNA staining of nuclear DNA from plant tissues in order to determine genome size variations. For this approximately 0.5 cm^2 of young and finally chopped leaf tissue was placed in a 55 mm plastic Petri dish. To this 400 µl of nuclei extraction buffer was added. The sample was filtered through a Partec 50 µm Cell Trics disposable filter and supernatant was collected. 1.6 ml of flprescent staining solution (DAPI) was added to the supernatant containing intact nuclei. The composite solution (Final suspension) was allowed to incubate for 30 to 60 seconds. The whole procedure was performed on ice.

Flow cytometry: Final suspension was analysed with CyFlow Ploidy Analyzer with UV-laser excitation (Partec, Germany). Flow cytometric analysis of DNA fluorescence intensity results in a distribution of relative fluorescence intensity, which is typical for given species and genotype (Kubaláková *et al*., 2002). At least ten thousand nuclei were analysed in each sample with three replications.

Data analysis : For the estimation of the size of the nuclear genome in *Brassica juncea*, *Solanum lycopersicum L.* cv. 'Stupicke´ polný´ rane´' with 2C nuclear DNA content=1.96 pg was used as an internal reference. Standard flow cytometry equation (Dolezel and Bartos, 2005) was used for estimating DNA content of the test samples to estimate DNA content as follows:

Sample 2C value (DNA pg) = Reference 2C value x sample 2C mean peak position/ reference 2C mean peak position

DNA size in pg can be converted into genome size (Mbp) by using following the formula as devised by Dolezel *et al*. (2007).

Number of base pairs = mass in pg x 0.978 x 10^9 or 1pg = 978 Mbp

Statistical software Minitab was used for the test of significance, standard deviation and correlation and regression analysis.

Results and Discussion

Flow cytometry is an efficient technique that is used primarily for the determination of ploidy levels, nuclear DNA content, genome size, and hybrid verification in plants (Bennett *et al*., 1995; Dolezel, 1997). The flow cytometric detection of ploidy is based on a simplified assumption that all the chromosomes have the same DNA content (Roux *et al*., 2001). Cytometric determination of nuclear DNA content is useful for studying the variation in DNA (Dolezel *et al*., 1989; Hammat *et al*., 1991) in plants. Several studies on variation in DNA content in polyploid populations (Kenton *et al*., 1986) show a decrease in DNA amount in subsequent generations. This technique has not been exploited in *Brassica* wide cross progenies. With increased interest in exploiting the wild crucifers to increase genetic variability and diversity, flow cytometry can be used effectively to analyze a large number of progenies to understand chromosome transmission, genic imbalance and ploidy variants (*e.g.* 3n, 2n + n).

We used a set of 36-advance generation progenies, derived from an interspecific cross (*B. fruticulosa*

x *B. juncea*) x *B. juncea*. 1C nuclear DNA content was estimated from five plants of each test accession from each replication as per the protocol described in material and methods. The IC DNA content of *B. juncea* introgression lines ranged between 1.124pg (AD4K2-196) and 1.269 (AD3L-526, AD3L-459) as compared to 1.220 pg recorded for the standard, *B. juncea* cv. RLC- 1(Table 1). Coefficient of variation ranged from 2-5%.The observed genome size was subsequently predicted

Table1: Nuclear 1C DNA content (pg) of recombinant inbred lines of *B. juncea*

Genotypes	1C DNA content in (pg)	DNA as percent of standard	Inferred genome size (Mbp)	Expected chromosome number	Differences in genome size (%)
AD3K-028	1.214	99.49	1187	35.81	-0.012
AD3K-030	1.216	99.72	1190	35.89	-0.007
AD3K-043	1.242	101.83	1215	36.65	0.045
AD4K2-196	1.124	92.16	1100	31.33	-0.191
AD3L-333	1.240	101.62	1213	36.59	0.040
AD3L-341	1.235	101.22	1208	36.43	0.030
AD3L-345	1.242	101.82	1215	36.65	0.044
AD3L-354	1.220	100.00	1193	36.00	0.000
AD3L-367	1.228	100.69	1201	36.24	0.017
AD3L-368	1.226	100.55	1200	36.19	0.013
AD3L-373	1.238	101.51	1211	36.54	0.037
AD3L-391	1.228	100.64	1201	36.23	0.016
AD3L-394	1.249	102.38	1222	36.85	0.058
AD3L-402	1.253	102.70	1225	36.97	0.066
AD3L-408	1.231	100.89	1204	36.32	0.022
AD3L-412	1.239	101.56	1212	36.56	0.038
AD3L-420	1.235	101.25	1208	36.45	0.031
AD3L-421	1.235	101.25	1208	36.45	0.031
AD3L-439	1.213	99.41	1186	35.78	-0.014
AD3L-446	1.188	97.39	1162	36.03	-0.064
AD3L-447	1.226	100.55	1200	36.19	0.013
AD3L-449	1.260	103.31	1233	37.19	0.081
AD3L-459	1.269	104.06	1242	39.54	0.099
AD3L-462	1.223	100.23	1196	36.08	0.006
AD3L-467	1.233	101.07	1206	36.38	0.026
AD3L-490	1.229	100.77	1202	36.27	0.019
AD3L-491	1.213	99.41	1186	35.78	-0.014
AD3L-494	1.235	101.25	1208	36.45	0.031
AD3L-502	1.228	100.65	1201	36.23	0.016
AD3L-526	1.269	104.06	1242	39.54	0.099
AD3L-533	1.241	101.71	1214	36.61	0.042
AD3L-539	1.223	100.23	1196	36.08	0.006
AD4K1-06	1.224	100.32	1197	36.11	0.008
AD4K1-099	1.199	98.31	1173	35.39	-0.041
AD4K1-096	1.210	99.18	1183	35.7	-0.020
AD4K1-190	1.231	100.94	1204	36.33	0.023
RLC-1	1.220	100.00	1193	36.00	0.000

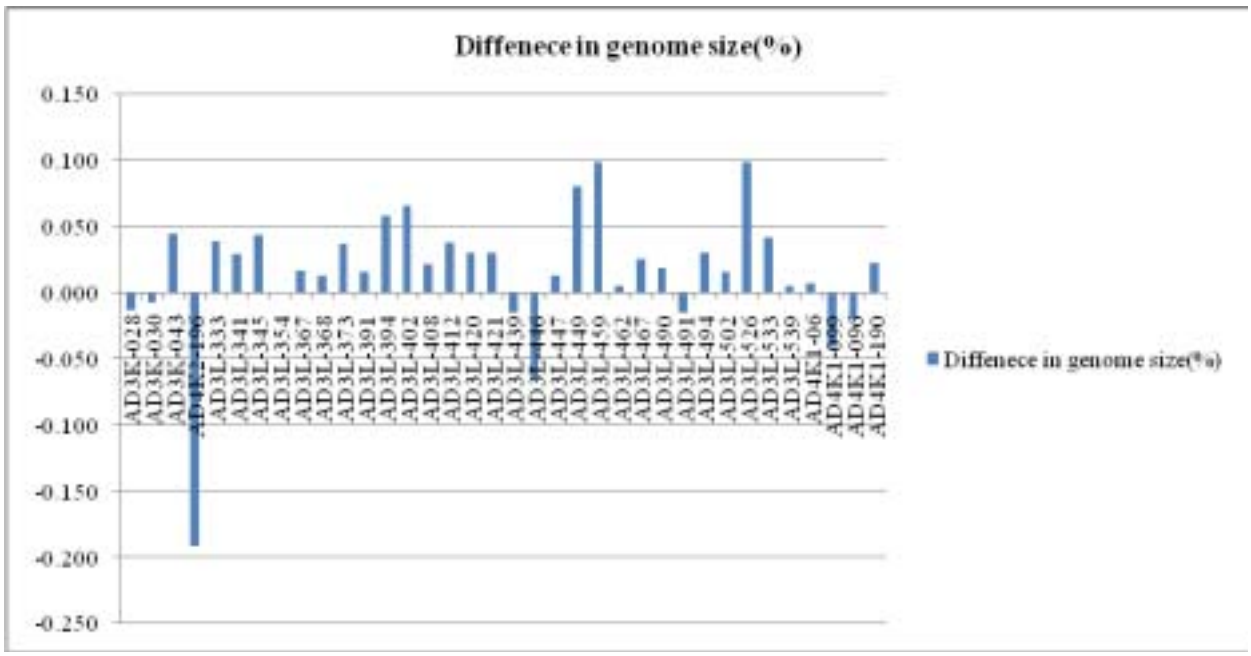

Fig.1 : Genome size variation in *B. juncea* introgression lines reflected as a percentage of check ,*B. juncea* cv RLC-1

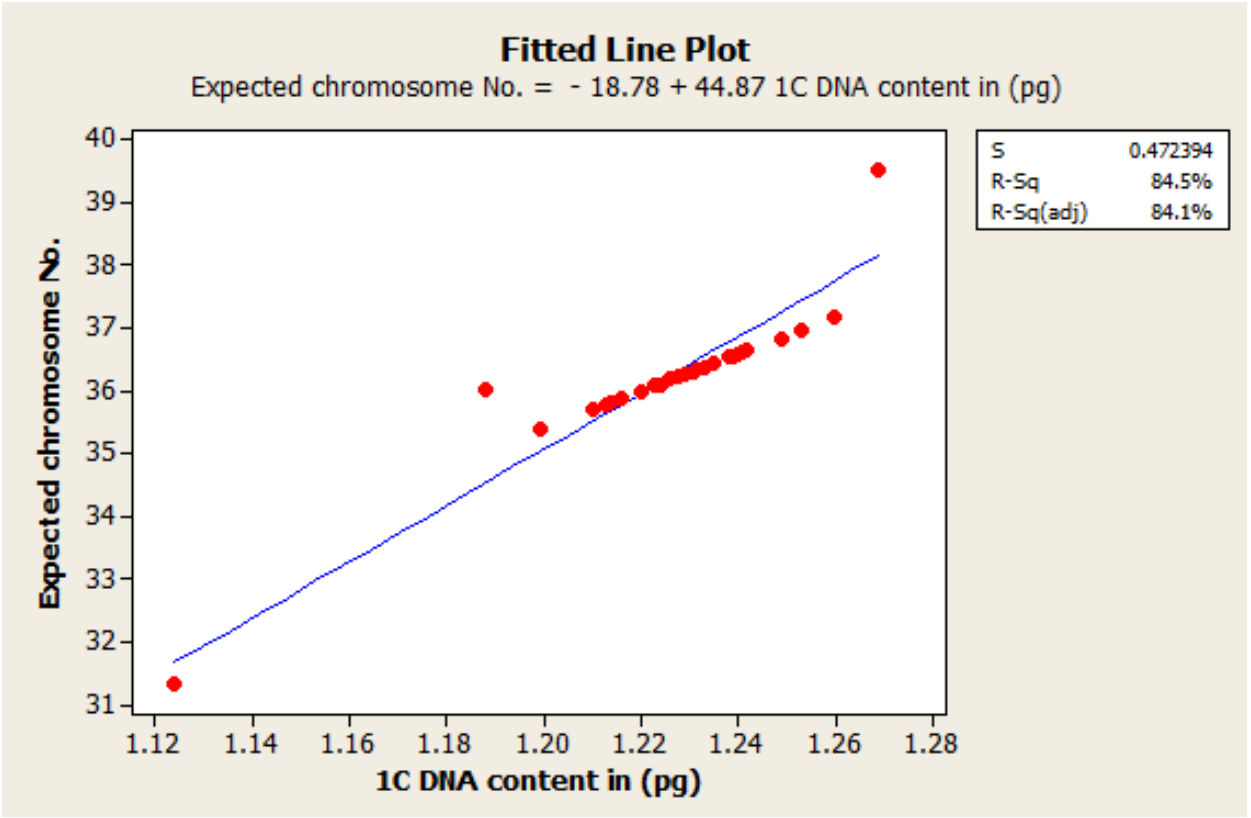

Fig 2: Regression lines between DNA content and expected chromosome number

by converting C value (pg) to base pairs using the formula as described earlier (Dodezel *et al*., 2007). The genome size in the introgressed progenies varied from 1100 Mbp to 1242 Mbp as depicted in Fig1. Our results clearly indicated a strong correlation between relative nuclear DNA content and the inferred chromosome number (Fig 2) with a high R^2 value of 0.846.

Increase in the genome size as compared to the standard *B. juncea* parent, RLC 1 may indicate the addition of alien chromatin from *B. juncea*. A reduction in genome size may indicate chromatin loss. This is a common outcome of duplication and deficiency in gametes in the introgressed progenies, during the fixation of introgression lines. Most plants determined by flow cytometry as euploid showed normal meiosis and high-pollen grain stainability. Current protocol is being improved to make it possible to detect addition or deletion of even a single chromosome.

We are now routinely using flow cytometry analyses to gain more insight into the variation and transmission of DNA content within in alien introgressed populations. Information on genome size and DNA content has practical significance for molecular characterization of populations with regard to the choice of primers and restriction enzymes, the construction of chromosome-specific libraries, and gene mapping (Bennett *et al*., 2000).

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